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Interspecies variability of propylene glycol dinitrate (PGDN)- induced methemoglobin formation was studied in vitro employing erythrocytes from four separate species. The net rate of methemoglobin formation was significantly different among species with dog>guinea pig>rat>human. This order of susceptibility was maintained in stroma-free hemolysates, indicating that interspecies variability was not a reflection of differences in red cell membrane permeability or intracellular transport of PGDN. The erythrocytic enzymes, catalase, superoxide dismutase, glutahione peroxidase, 6-phosphogluconate dehydrogenase, glucose-6-phosphate dehydrogenase, methemoglobin reductase, and glutathione-S-transferase, were assayed by adaption of existing methods to a centrifugal analyzer. The above enzymes were removed from hemoglobin derived from each species and the order of susceptibility to PGDN-induced methemoglobin formation remained essentially the same with dog>guinea pig>human = rat. However, the net rate of PGDN-mediated oxidation of hemoglobin to methemoglobin increased in purified hemoglobin preparations from each species. These results demonstrate (See reverse)

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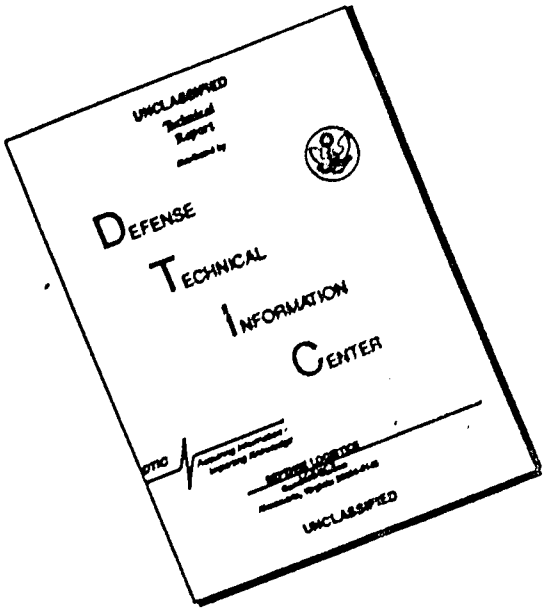

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19. Abstract (continued) that there is species variability in the net rate of PGDN-mediated methemoglobin formation. Total enzyme activity in erythrocytes may contribute to reduction in the net rate of methemoglobin formation. However, the primary determinant of the net rate of methemoglobin formation induced by PGDN appears to be the structure of each hemoglobin molecule.

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Interspecies Variability in Propylene Glycol Dinitrate-Induced Methemoglobin Formation[1]

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Propylene glycol dinitrate (PGDN, 1,2-propanediol dinitrate) is the primary component of the Naval torpedo propellant, Otto Fuel II. Symptoms of PGDN intoxication, including vasodilation, hypotension, headache, and methemoglobin formation (Jones *et al.*, 1972; Stewart *et al.*, 1974), are similar to those caused by other nitrated esters (Ellis *et al.*, 1984; Strein *et al.*, 1984; Andersen and Mehl, 1973; Clark and Litchfield, 1969; Carmichael and Lieben, 1963). Acute intoxication of animals with PGDN produces methemoglobinemia, and the resulting anoxia is proposed as the immediate cause of death (Clark and Litchfield, 1969; Jones *et al.*, 1972; Andersen and Mehl, 1973).

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The specific mechanism of the reaction of nitrate esters with hemoglobin is quite complex and as yet not well understood. Oxidation of human and rat hemoglobin by PGDN has been attributed to direct interaction of the dinitrate with oxyhemoglobin (Andersen and Smith, 1973). Other nitrate esters produce methemoglobin by cleavage of nitrite from the ester, followed by hemoglobin oxidation by nitrite (Strein *et al.*, 1984). The reaction of PGDN with hemoglobin does release nitrite within the erythrocyte (Clark and Litchfield, 1969) and this nitrite may further stimulate methemoglobin formation. Moreover, denitrification of nitrate esters may occur enzymatically through glutathione-*S*-transferase or nonenzymatically by reaction with glutathione (Heppel and Hilmoe, 1950; Needleman and Hunter, 1965; Chasseaud *et al.*, 1978). Mechanisms potentially exist for both direct interaction of PGDN with hemoglobin and nitrite oxidation of hemoglobin to produce methemoglobin with this nitrate ester. Once initiated by nitrite, oxidation of hemoglobin to methemoglobin proceeds autocatalytically as a result of superoxide production (Watanabe and Ogata, 1981; Kosaka *et al.*, 1979; Misra and Fridovich, 1972; Lynch *et al.*, 1976, 1977).

There is *in vivo* species variability in the rate of methemoglobin formation following PGDN exposure, with dogs and monkeys being more susceptible than rats or guinea pigs (Jones *et al.*, 1972). Similarly, interspecies variability was reported for nitrite-induced methemoglobin formation in isolated erythrocytes from rats, sheep, and humans (Calabrese *et al.*, 1983). Both of the above studies suggested that the observed differences in susceptibility to methemoglobin formation may result from variable activities of erythrocyte methemoglobin reductase. Indeed, human erythrocytes congenitally impaired by insufficient methemoglobin reductase activity often contain elevated methemoglobin amounts (Scott, 1960). Factors other than total methemoglobin reductase enzyme activity in erythrocytes may also contribute to observed species variability in net rates of hemoglobin oxidation. It is possible that antioxidants in erythrocytes from different species, including amounts of antioxidant enzymes, may contribute to reduction in the net rate of methemoglobin formation. Also, variations in amounts of glutathione-*S*-transferase may be important in releasing nitrite from nitrate esters, subsequently affecting net oxidation rates. Finally, species variation in hemoglobin structure may contribute to the variability of PGDN-mediated oxidation of hemoglobins.

In this study we determined if variability existed in the net rate of PGDN-mediated formation of methemoglobin in isolated erythrocytes from four mammalian species. In addition, cell-free and partially purified hemoglobin preparations from each species were examined to determine if net rates of methemoglobin production varied among species. Selected enzyme activities in erythrocytes from each species were also compared in an attempt to explain variations observed in PGDN-mediated hemoglobin oxidation. Enzyme profiles plus results with cell-free and partially purified hemoglobin preparations were examined to determine the relative contribution of enzymes and the hemoglobin molecule itself in maintenance of oxyhemoglobin upon exposure to PGDN.

METHODS

Chemicals

PGDN was graciously provided by Mr. Robert Sarncomb, Naval Surface Weapons Center, Indian Head, Maryland.

PGDN was stored as a 10% solution in ethanol until needed, at which time 1 to 3 ml of PGDN was separated from ethanol by extraction of ethanol in 10 to 30 ml water. The purity of PGDN isolated in this matter was verified by gas–liquid chromatography employing an electron capture detector (Erk *et al.*, 1982).

Tris(hydroxymethyl)aminomethane and 1-chloro-2,4-dinitrobenzene were purchased from Aldrich Chemical Company, sodium citrate was obtained from J. T. Baker, and disodium ethylenediaminetetraacetate (EDTA) was from Fisher Scientific. β-D-Glucose 6-phosphate, β-NAD$^+$, β-NADP$^+$, β-NADPH, cysteine, glutathione reductase, glycylglycine, maleimide, phosphogluconic acid, pyrogal-

lol, and sodium azide were purchased from Sigma Chemical Company. All other chemicals used in this study were of reagent-grade quality.

Blood Collection

Fresh blood was obtained from Fisher 344 rats and Hartley guinea pigs by cardiac puncture, under ether anesthesia, and from the jugular veins of beagle dogs. All animals were housed in cages with water and laboratory chow provided *ad libitum*. Human blood was collected from brachial veins of volunteers within our laboratory. The anticoagulant used in all collections was citrate–phosphate–dextrose (Lee and Henry, 1979).

Erythrocyte Isolation and Hemoglobin Preparation

Erythrocytes were separated from plasma by centrifugation and washed three times in Dulbecco's phosphate-buffered saline (Dulbecco and Vogt, 1954) or physiological saline. Hemolysates were prepared by lysing red cells in an equal volume of 1 mM phosphate buffer (pH 7.8). Crystallization of hemoglobin solutions was prevented by adjusting the pH to 7.8 (dog, guinea pig, and human) or 8.5 (rat). Red cell stroma was removed by centrifugation at 30,000*g* max for 30 min.

Partial purification of dog, guinea pig, and human hemoglobins was accomplished by ion-exchange chromatography with carboxymethyl cellulose cation exchange resin, Whatman CM 52 (Lynch *et al.*, 1977). Stroma-free hemoglobin solutions were dialyzed overnight against 2 liters of distilled water. The dialysate (approximately 20 to 40 ml) was diluted to 500 ml with 5 mM phosphate buffer (pH 6.8) and added to a column containing CM 52 resin which had been equilibrated with the same phosphate buffer (bed volume approximately 75 to 125 ml). Under these conditions the hemoglobin readily bound to the resin with only small amounts eluting off the column. The column was developed overnight with 5 mM phosphate buffer to ensure a clear column effluent after which hemoglobins were eluted from the resin with 1 M phosphate buffer (pH 6.8). The hemoglobin effluent was collected, the pH adjusted to 7.8 with 1 N NaOH, and the solution concentrated by ultrafiltration using an Amicon stirred cell concentrator with a PM 30 membrane filter.

Rat hemoglobin crystallized at neutral pH, preventing purification of this hemoglobin by the method employed for the other species. Therefore, 30,000*g* max supernatant fractions from rat hemolysates were adjusted to pH 7 to allow crystallization. Solutions were stored in an ice bath for approximately 2 hr, after which crystals were collected by centrifugation at 500*g*. The supernatant fraction was decanted, and the crystalline pellet was resuspended and washed in an equal volume of distilled water (three repetitions). Crystals of rat hemoglobin were dissolved by adjusting the pH to 9.3 with 1 N NaOH, and insoluble material was removed by centrifugation at 30,000*g* max for 10 min. Resultant supernatant fractions were dialyzed overnight against distilled water.

In Vitro Incubations

Erythrocytes, hemolysates, and partially purified hemoglobin preparations were incubated in polypropylene tubes at 37°C in a shaking water bath. The hemoglobin concentration for all incubations was approximately 775 μM (total volume = 5 ml). Amounts of PGDN were measured gravimetrically (*d* = 1.6 g/ml at 20°C) and added neat (10 mM) or in dilution with ethanol (3, 1, and 0.3 mM), with control preparations receiving ethanol only. Samples (0.2 ml) were removed from erythrocyte–PGDN mixtures at specified times during the 3 hr incubations and lysed in 0.8-ml volumes of 1 mM phosphate buffer (pH 7.8). From these hemolysates and from partially purified hemoglobin solutions, 0.3-ml aliquots were removed, diluted in 0.4 ml of 50 mM phosphate buffer (pH 6.6), and assayed for methemoglobin as described below.

Analytical Methods

Spectrophotometric assays, with the exception of the catalase enzyme assay, employed a Cobas-Bio centrifugal analyzer (Roche Analytical Instruments, Inc.). Assays were adapted to the centrifugal analyzer from previously reported methods. Unless otherwise stated, reagents were prepared in distilled water and concentrations listed represent the final concentration in the reaction cuvette. The specific program parameters employed are listed in Table 1. The concentration of hemoglobin in samples assayed for enzymatic activity was initially about 50 mg/ml. The specific dilutions prepared for each assay are described below.

Hemoglobin and methemoglobin. The concentration of hemoglobin in whole-cell preparations, in hemolysates, and in partially purified hemoglobin solutions was determined with Drabkins reagent (van Kampen and Zijlstra, 1961). Prior to assay washed whole-cell preparations were lysed by osmotic shock by diluting 0.1 ml red cells with 0.9 ml 1 mM phosphate buffer (pH 7.8).

Methemoglobin was determined by modifying the method of Evelyn and Malloy (1938) to permit use of the centrifugal analyzer. The optical density (630 nm) of hemoglobin solutions was measured with four separate reagents. All reagents (total volume 10 ml) contained 9.375 ml 0.1 M phosphate buffer (pH 6.6); reagents 1 and 3 contained 0.625 ml distilled water, while reagents 2 and 4 contained 0.625 ml 0.4 M $K_3Fe(CN)_6$. Finally, 0.61 mmol KCN was dissolved in reagents 3 and 4. Total hemoglobin concentrations were adjusted to about 0.5 μM with 50 mM phosphate (pH 6.6) or 1 mM phosphate (pH 7.8) for rat, and analysis was performed with each reagent by the pro-

TABLE I

PARAMETER LISTING FOR ENZYMATIC ACTIVITY ASSAYS PERFORMED ON THE COBAS-BIO CENTRIFUGAL ANALYZER

Parameter	Cyanomet-hemoglobin (mol/l)	Methemoglobin (mmol/l)	GSH peroxidase (mol/l)	GSH-*S*-transferase (mol/l)	Glucose-6-phosphate dehydrogenase (mol/l)	6-Phosphogluconate dehydrogenase	Methemoglobin reductase (U/l)
Calculation factor	227.27	1	2009.6	1041.67	1607.7	1607.7	238.1
Limit	500	3	1	2	1	1	2
Temperature (°C)	25	25	25	25	30	25	37
Type of analysis	1	5	3	3	2	2	3
Wavelength	540	630	340	340	340	340	575
Sample volume (μl)	25	25	20	25	25	25	25
Diluent volume (μl)	25	25	20	30	25	25	25
Reagent volume (μl)	250	200	250	150	200	200	170
Incubation time (sec)	10	0	10	10	0	0	600
Starting reagent volume (μl)	0	0	10	25	0	0	10
Time of first reading (sec)	240	180	10	1	10	10	0.5
Time interval (sec)	240	180	10	10	10	10	10
Number of readings	2	1	9	24	10	10	30
Blanking mode	1	1	1	1	1	1	1
Printout mode	1	3	4, 1	1	1, 3, 4	1, 3	1

gram parameters listed in Table 1. The percentage of methemoglobin was calculated by

$$\% \text{ metHb} = 100\left[\frac{OD_1 - OD_3}{OD_2 - OD_4}\right]$$

where metHb = methemoglobin and OD = optical density. The net percentage of methemoglobin formed by PGDN oxidation of hemoglobin was calculated by subtracting the percentage of methemoglobin in control (ethanol-treated) hemoglobin from that of PGDN-treated hemoglobins.

Catalase activity. Catalase was determined by existing methods (Beers and Sizer, 1952; Aebi, 1971). Samples were diluted with 0.1 M phosphate buffer (pH 7.0) so that a ΔOD_{240} from 0.45 to 0.40 occurred in approximately 10 sec (Beckman DK2 uv-vis spectrophotometer). The dilution ratio for dog was 1:10, for the rat it was 1:20, and for guinea pig and human it was 1:30.

Superoxide dismutase (SOD). CuZn SOD activity was measured employing a modified pyrogallol autoxidation method (Gray *et al.*, 1985).

Glutathione peroxidase. Samples were diluted in an equal volume of 0.15 M phosphate buffer (pH 7.0) containing EDTA (0.15 M final concentration). Hemoglobin was oxidized to methemoglobin by adding 4 parts Drabkins reagent about 10 min prior to beginning the assay. The reagent mixture consisted of 50 mM phosphate buffer, 5 mM EDTA (pH 7.0), 3.7 mM sodium azide, 5 mM reduced glutathione (prepared in 50 mM phosphate buffer, pH 7.0), 1 eU glutathione reductase, and 0.28 mM NADPH. Hydrogen peroxide (70 μM) was used to start the reaction (Paglia and Valentine, 1967).

Glutathione-S-transferase. Samples were diluted in equal volumes of 1 mM phosphate buffer (pH 7.5). The reagent mixture consisted of 0.75 mM 1-chloro-2,4-dinitrobenzene (prepared in ethanol) and 0.1 M phosphate buffer (pH 6.25). Reduced glutathione was added to start the reaction (Carmagnol *et al.*, 1981).

Glucose-6-phosphate dehydrogenase. Samples prepared from dog, guinea pig, and human hemolysates were diluted with equal volumes of 10 mM Tris buffer (pH 7.5). Rat hemolysates were diluted with 4 vol of 10 mM Tris (pH 7.5). Enzyme activity was assayed utilizing the method of Kachmar and Moss (1976), with a reagent mixture composed of 0.1 M Tris buffer (pH 7.5), 10 mM $MgCl_2 \cdot 6H_2O$, 0.2 mM NADP, 0.7 mM β-D-glucose 6-phosphate, and 4 mM maleimide.

6-Phosphogluconate dehydrogenase. Diluted samples prepared for the glucose-6-phosphate dehydrogenase assays were used for this determination. The assay used a reaction mixture composed of 50 mM glycylglycine buffer (pH 8.6), 20 mM $MgCl_2$, 2 mM phosphogluconic acid, 6.5 mM cysteine, and 1.5 mM NADP (King, 1971).

Methemoglobin reductase. The dilution ratio for samples, with 1 mM phosphate buffer (pH 7.8), was 1:5 for dog, 1:10 for rat and human, and 1:20 for guinea pig. The reagent mixture for this assay consisted of 0.54 mM EDTA (disodium salt), 5 mM sodium citrate (trisodium salt), 0.16 mM $K_3Fe(CN)_6$, and 3.5 μM diaphorase-free hemoglobin from rat. NADH (0.2 mM) prepared in 1 mM phosphate buffer (pH 7.8) was used as the starter reagent. The assay was adapted from the method of Hagesh *et al.* (1968).

Statistical Analysis

Amounts of PGDN-induced methemoglobin formation in erythrocytes, hemolysates, and partially purified hemoglobins from different species were evaluated for significant differences by two- and three-factorial multivariate repeated-measure designs (Dixon, 1983; Timm, 1975). The factors included species, dose, and type of preparation with time being the repeated measure. A pairwise Scheffé test was used to analyze differences within given significant interactions.

RESULTS

In vitro comparison of the relative rates of PGDN-induced methemoglobin formation in intact erythrocytes from dog, guinea pig, rat, and human demonstrated interspecies differences (Fig. 1). At a concentration of 10 mM PGDN the amount of methemoglobin formation in dog erythrocytes was significantly higher ($p < 0.05$) than that of human erythrocytes after 1 hr incubation. After 2 hr, dog methemoglobin was significantly higher than

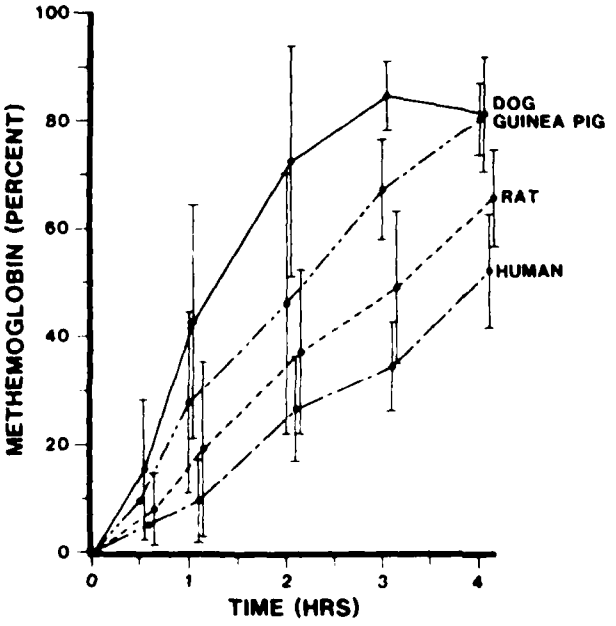

FIG. 1. PGDN-induced methemoglobin formation in erythrocytes from different species. Concentration of PGDN 10 mM. Values represent the $\bar{x} \pm$ standard deviation, and individual data points for each time period are offset for purposes of presentation. See Methods for specific details about erythrocyte preparations.

for hemoglobin of all other species. At 3 hr, methemoglobin content of guinea pig was similar to that of dog and different from that of human and rat. Methemoglobin quantities in rat erythrocytes were significantly higher than human after 4 hr ($p < 0.001$) but remained below that observed for dog and guinea pig. Under these experimental conditions, the net rate of PGDN-induced methemoglobin formation was dog > guinea pig > rat ≥ human. The relatively high amount of methemoglobin formation in dogs has been previously reported *in vivo*, following chronic inhalation of PGDN vapors (Jones *et al.*, 1972).

The net rate of methemoglobin formation was compared in stroma-free hemolysates to evaluate whether differences in oxidation of hemoglobins might be attributable to differences in the membrane permeability of various red cells to PGDN. Figure 2 shows methemoglobin formation as a function of time for hemolysates (30,000*g* supernatant fractions prepared from erythrocytes from each species). The concentration of PGDN tested with 775 μM hemoglobin was 3 mM. Significant differences among species were observed at different times during the incubation. Thirty minutes after the addition of PGDN, the net amounts of methemoglobin was significantly higher in dog and guinea pig as compared to that of human and rat hemolysates. Differences between the amounts of methemoglobin formed for human and rat hemolysates were statistically significant after 3 hr, while differences between dog and guinea pig were significant after 4 hr incubation. Thus, after 4 hr net methemoglobin formation was significantly different in hemolysates prepared from each species with dog > guinea pig > rat > human. The order of susceptibility to methemoglobin formation observed with intact erythrocytes was maintained in hemolysate preparations.

Methemoglobin formation in hemolysates from each species was a function of PGDN concentration (Fig. 3). Significant differences among species at specific times during an incubation were dependent upon the concentration of PGDN employed. Human hemoglobin was the least susceptible to oxidat on by PGDN when the dose was increased.

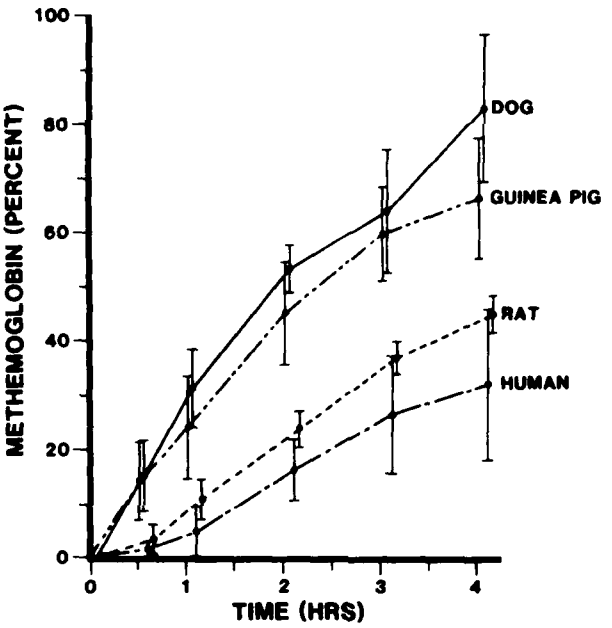

FIG. 2. PGDN-induced methemoglobin formation in stroma-free hemolysates from different species. Concentration of PGDN = 3 mM. Values represent the $\bar{x} \pm$ standard deviation, and individual data points for each time period are offset for purposes of presentation. See Methods for specific details regarding preparation of hemolysates.

Table 2 lists the enzyme activity determined for erythrocytes from each of the four species. Species with high methemoglobin reductase activity are not consequently better protected against hemoglobin oxidation by PGDN (Jones *et al.*, 1972). Methemoglobin reductase activity is three times higher in guinea pig erythrocytes compared to that of human and rat, although the latter were significantly less susceptible to methemoglobin formation (Figs. 1 and 2). Second, glutathione-*S*-transferase activity in dog and guinea pig erythrocytes is lower than that determined for rat and human. Also, rat erythrocytes possess a more active complement of antioxidant enzymes, including glutathione peroxidase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and superoxide dismutase, as compared with other species. These results suggest that rat erythrocytes may be better protected against the oxidation of hemoglobin to methemoglobin. The lack of measurable

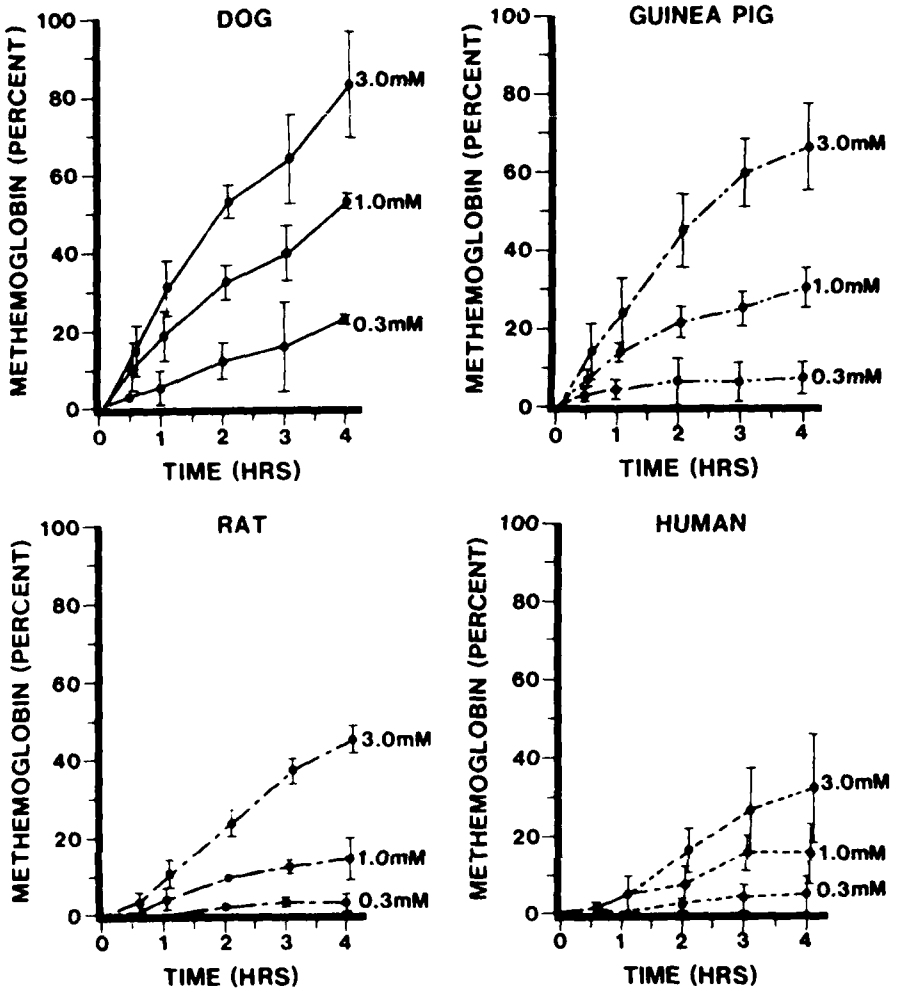

FIG. 3. Formation of methemoglobin in stroma-free hemolysates from different species at varying PGDN concentrations. Values represent the $\bar{x} \pm$ standard deviation, and individual data points for each time period are offset for purposes of presentation. See Methods for specific details.

catalase activity in dog erythrocytes is consistent with this suggestion. In conflict with this suggestion is the finding that the antioxidant enzyme profile of human erythrocytes resembles that of the guinea pig, even though human hemoglobin preparations are relatively resistant to oxidation by PGDN.

Assays of the rate of oxidation of partially purified hemoglobin solutions from each species were performed with 1 mM PGDN to determine the effect of absence of these enzymes on methemoglobin formation. There was no detectable enzyme activity in hemoglobin preparations prepared by column chromatography as described above. In addition to the removal of enzymes, partial purification of hemoglobin eliminated small molecules which allosterically affect stability within erythrocytes. Removal of these compounds may have influenced the extent of oxidation of individual hemoglobin molecules. The amount of methemoglobin formation in these partially purified hemoglobin preparations appears elevated for all species, compared to that observed for hemolysates (Fig. 4). Differences were statistically significant only for dog and guinea pig. Therefore, those species most susceptible to PGDN-induced methemoglobin formation appear to be afforded the greatest degree of protection by their erythrocytic enzyme complement. Also, the order of susceptibility observed in hemolysates, as well as whole cells, is not altered by the removal of erythrocytic enzymes.

TABLE 2

COMPARATIVE ENZYME ACTIVITY IN ERYTHROCYTES FROM DIFFERENT SPECIES[a]

Enzyme	Animal species			
	Dog (n = 8)[b]	Guinea pig (n = 7)	Human (n = 8)	Rat (n = 6)
Methemoglobin reductase (μmol/min/g Hb[c])	2.18 ± 0.82[d]	15.5 ± 1.88	4.66 ± 1.29	3.59 ± 0.90
Glutathione-S-transferase (μmol/min/mg Hb)	0.73 ± 0.24	2.57 ± 0.83	3.88 ± 1.28	3.86 ± 1.32
Catalase (μmol/min/mg Hb)	ND[e]	133 ± 40	188 ± 31	121 ± 33
Superoxide dismutase (unit/mg Hb)	2.39 ± 0.44	1.66 ± 0.41	1.90 ± 0.34	3.98 ± 0.31
Glutathione peroxidase (μmol/min/g Hb)	104 ± 20	66 ± 16	18 ± 9	606 ± 182
6-Phosphogluconate dehydrogenase (μmol/min/mg Hb)	2.64 ± 0.62	3.07 ± 0.51	3.85 ± 0.29	5.25 ± 3.02
Glucose-6-phosphate dehydrogenase (μmol/min/mg Hb)	5.31 ± 0.99	5.42 ± 1.04	5.46 ± 1.54	19.1 ± 6.45

[a] Activity for enzymes (except catalase) was determined with a Cobas-Bio centrifugal analyzer (Roche Analytical Instruments). Catalase activity was determined at 240 nm in a Beckman DK-2 spectrophotometer.

[b] *n* = number of determinations.

[c] Hb = hemoglobin

[d] Values represent the $\bar{x}$ ± standard deviation.

[e] ND = not detected.

DISCUSSION

PGDN reacts with oxyhemoglobin in solution, resulting in oxidation of the molecule to methemoglobin. The rate of methemoglobin formation in hemoglobin solutions upon addition of PGDN reflects a direct interaction with oxyhemoglobin, oxidation of hemoglobin by nitrite released from PGDN, and autocatalytic oxidations due to active oxygen. These reactions collectively produce the methemoglobin observed upon addition of PGDN. Enzymatic cleavage by glutathione-S-transferase of PGDN with release of nitrite is not required to initiate methemoglobin formation in hemoglobin preparations.

Interspecies variability in PGDN-mediated methemoglobin formation has previously been reported *in vivo* (Jones *et al.*, 1972). The variability among species *in vitro*, demonstrated in the present investigation, indicates that factors responsible for varied susceptibility to methemoglobin formation are primarily attributed to factors associated with the erythrocyte. In partially purified hemoglobin solutions, stroma-free hemolysates, and erythrocytes, the net rate of PGDN-mediated methemoglobin formation follows the sequence dog > guinea pig > rat ≥ human. Thus, effects of variation in erythrocyte membrane permeability to PGDN among species does not account for varied susceptibility to methemoglobin formation. The observed decrease in net amounts of methemoglobin formed in dog and guinea pig stroma-free hemolysates compared to partially purified hemoglobin solutions probably reflects the contribution of enzymes and/or small molecular weight allosteric affectors present in the latter preparations. Although there was a similar, slight decrease in methemoglobin formation in human and rat stroma-free hemo-

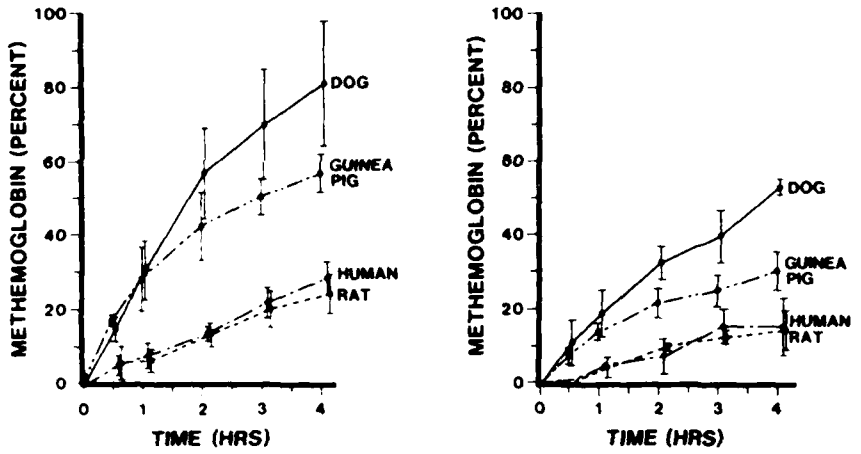

FIG. 4. Comparison of methemoglobin formation in enzyme-free hemoglobin solutions and stroma-free hemolysates from different species. Concentration of PGDN = 1 mM. Values represent the $\bar{x} \pm$ standard deviation, and individual data points for each time period are offset for purposes of presentation. See Methods for specific details.

lysates compared to hemoglobin solutions, the decrease was not statistically significant. Thus, the contribution of antioxidant enzymes in human and rat stroma-free hemolysates does not appear to alter net methemoglobin formation substantially, even though the rat erythrocyte is comparatively well-equipped to handle oxidant stress. However, incubation mixtures were not fortified with excess reductants and cofactors which, upon depletion from the reaction mixtures, may have limited the antioxidant enzyme activity. Further studies are required to outline contributions of specific enzymes, as well as allosteric stabilizers, to maintenance of oxyhemoglobin amounts in erythrocytes.

The primary determinant of the relative susceptibility of different species to PGDN-induced hemoglobin oxidation appears to be the molecular structure of each hemoglobin. Maintenance of the order of susceptibility to methemoglobin formation in each type of preparation, erythrocyte, stroma-free hemolysate, and enzyme-free hemoglobins, lends support to specific hemoglobin structure controlling PGDN-mediated oxidation rates. Differences in hemoglobin structure reflect differences in globin chains of the molecule since heme moieties are identical. That structural differences among animal hemoglobins result in different susceptibilities to oxidation is somewhat analogous to observations with abnormal human hemoglobins of the M and S types, which, because of congenital alterations in the amino acid sequence of globin chains, are more labile to oxidation. The results of this study point out the need for careful selection of an animal model when studying toxicant-induced oxidation of hemoglobin. Assuming nitrate esters, in general, behave like PGDN, the animal model most closely resembling man is rat.

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